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| (54) Title: IN VITRO GROWTH OF FUNCTIONAL ISLETS OF LANGERHANS AND IN VIVO USES THEREOF (57) Abstract The subject invention concerns new methods which make it possible, for the first time, to grow functional islets in <i>in vitro</i> cultures. The subject invention also concerns the use of the <i>in vitro</i> grown islet-like structures for implantation into a mammal for <i>in vivo</i> therapy of diabetes. The subject invention further concerns a process using the <i>in vitro</i> grown islet implants for growing an organ <i>in vivo</i> that has the same functional, morphological and histological characteristics as those observed in normal pancreatic tissue. The ability to grow these cells <i>in vitro</i> and organs <i>in vivo</i> opens up important new avenues for research and therapy relating to diabetes. | | |

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DESCRIPTIONIN VITRO GROWTH OF FUNCTIONAL ISLETS OF
LANGERHANS AND IN VIVO USES THEREOF

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Background of the Invention

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Diabetes is a major public health problem. As presented in the 1987 Report of The National Long-Range Plan to Combat Diabetes commissioned by the National Diabetes Advisory Board, six million persons in the United States are known to have diabetes, and an additional 5 million have the disease which not yet been diagnosed. Each year, more than 500,000 new cases of diabetes are identified. In 1984, diabetes was directly causal in 35, 000 American deaths and was a contributing factor in another 95,000.

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Ocular complications of diabetes are the leading cause of new cases of legal blindness in people ages 20 to 74 in the United States. The risk for lower extremity amputation is 15 times greater in individuals with diabetes than in individuals without it. Kidney disease is a frequent and serious complication of diabetes. Approximately 30 percent of all new patients in the United States being treated for end-stage renal disease have diabetes. Individuals with diabetes are also at increased risk for periodontal disease. Periodontal infections advance rapidly and lead not only to loss of teeth but also to compromised metabolic function. Women with diabetes risk serious complications of pregnancy. Current statistics suggest that the mortality rates for infants of mothers with diabetes is approximately 7 percent.

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Clearly, the economic burden of diabetes is enormous. Each year, patients with diabetes or its complications spend 24 million patient-days in hospitals. A conservative estimate of total annual costs attributable to diabetes is at least \$24 billion (American Diabetes Association est., 1988); however, the full economic impact of this disease is even greater because

5 additional medical expenses often are attributed to the specific complications of diabetes rather than to diabetes itself.

Diabetes is a chronic, complex metabolic disease that results in the inability of the body to properly maintain and use carbohydrates, fats, and proteins. It results from the interaction of various hereditary and
10 environmental factors and is characterized by high blood glucose levels caused by a deficiency in insulin production or an impairment of its utilization. Most cases of diabetes fall into two clinical types: Type I, or juvenile-onset, and Type II, or adult-onset. Type I diabetes is often referred to as Insulin Dependent Diabetes, or IDD. Each type has a different
15 prognosis, treatment, and cause.

Approximately 5 to 10 percent of diabetes patients have IDD. IDD is characterized by a partial or complete inability to produce insulin usually due to destruction of the insulin-producing β cells of the pancreatic islets of Langerhans. Patients with IDD would die without daily insulin injections to
20 control their disease.

Few advancements in resolving the pathogenesis of diabetes were made until the mid-1970s when evidence began to accumulate to suggest that Type I IDD had an autoimmune etiopathogenesis. It is now generally accepted that IDD results from a progressive autoimmune response which
25 selectively destroys the insulin-producing β cells of the pancreatic Islets of Langerhans in individuals who are genetically predisposed. Autoimmunity to the β cell in IDD involves both humoral (Baekkeskov *et al.*, 1982; Baekkeskov *et al.*, 1990; Reddy *et al.*, 1988; Pontesilli *et al.*, 1987) and cell-mediated (Reddy *et al.*, 1988, *supra*; Pontesilli *et al.*, 1987, *supra*; Wang *et al.*, 1987) immune mechanisms. Humoral immunity is
30 characterized by the appearance of autoantibodies to β cell membranes (anti-69 kD and islet-cell surface autoantibodies), β cell contents (anti-carboxypeptidase A₁, anti-64 kD and/or antiGAD autoantibody), and/or β

5 cell secretory products (anti-insulin). While serum does not transfer IDD, anti- β cell autoantibody occurs at a very early age, raising the question of an environmental trigger, possibly involving antigenic mimicry. The presence of cell-mediated immunological reactivity in the natural course of IDD is evidenced by an inflammatory lesion within the pancreatic islets, termed
10 insulinitis. Insulinitis, in which inflammatory/immune cell infiltrates are clearly visible by histology, has been shown to be comprised of numerous cell types, including T and B lymphocytes, monocytes and natural killer cells (Signore *et al.*, 1989; Jarpe *et al.*, 1991). Adoptive transfer experiments using the NOD (non-obese diabetic) mouse as a mode of human IDD have
15 firmly established a primary role for auto-aggressive T lymphocytes in the pathogenesis of IDD (Bendelac, *et al.*, 1987; Miller *et al.*, 1988; Hanafusa *et al.*, 1988; Bendelac *et al.*, 1988). Unfortunately, the mechanisms underlying destruction of the pancreatic β cells remain unknown.

Recent efforts to culture pancreatic cells, including efforts reported
20 in the following publications, have focused on cultures of differentiated or partially differentiated cells which in culture have grown in monolayers or as aggregates. By contrast to these reports, the instant invention discloses a method and a structure wherein an islet-like structure is produced which has a morphology and a degree of cellular organization much more akin to a
25 normal islet produced *in vivo* through neogenesis.

Gazdar *et al.*, (1980) disclosed a continuous, clonal, insulin- and somatostatin-secreting cell line established from a transplantable rat islet cell tumor. However, the cells disclosed were tumorigenic and were not pluripotent.

30 Brothers, A.J. (WO 93/00441, 1993) disclosed hormone-secreting cells, including pancreatic cells, maintained in long-term culture. However, the cells cultured are differentiated, as opposed to pluripotent stem cells,

5 which are selected at an early stage for their hormone secreting phenotype.
as opposed to their capacity to regenerate a pancreas-like structure.

Korsgren *et al.*, disclosed an *in vitro* screen of compounds for their potential to induce differentiation of fetal porcine pancreatic cells. The instant invention does not depend on the use of fetal tissue.

10 Nielsen. J.H., (WO 86/01530, 1986) disclosed a method for proliferation of wholly or partially differentiated beta cells. However, this disclosure depended on fetal tissue as a source of the islet cells grown in culture.

15 McEvoy *et al.* (1982), disclosed a method for tissue culture of fetal rat islets and compared the effect of serum on the defined medium maintenance, growth and differentiation of A, B, and D cells. Once again, the source of islet cells is fetal tissue.

20 Zayas *et al.* (EP 0 363 125, 1990), disclosed a process for proliferation of pancreatic endocrine cells. The process depends on the use of fetal pancreatic tissue, and a synthetic structure, including collagen must be prepared to embed these cells for implantation. The thus produced aggregates of cultured cells upon implantation require 60-90 days before having any effect on blood glucose levels. and require 110-120 days before euglycemia is approached. By contract, the instant invention provides *in vitro* grown islet-like structures which do not require collagen or other
25 synthetic means for retention of their organization, in which, upon implantation, provide much more rapid effects on the glycemic state of the recipient.

30 Coon *et al.* (WO 94/23572, 1994), disclosed a method for producing an expanded, non-transformed cell culture of pancreatic cells. Aggregated cultured cells are then embedded in a collagen matrix for implantation, with the attendant shortcomings notes for the Zayas *et al.*, *supra* structures and

5 the distinctions noted with the structure produced according to the instant invention.

Despite the foregoing reports, the instant invention, wherein functional islet-like structures containing cells which express insulin, glucagon and/or somatostatin which can be implanted into clinically
10 diabetic mammals which subsequently remain healthy (after elimination of insulin treatment), is surprising. This is because conventional and immunofluorescent histology of the pancreatic islets of Langerhans (Lacey *et al.*, 1957; Baum *et al.*, 1962; Dubois, 1975; Pelletier *et al.*, 1975; Larsson
15 *et al.*, 1975), together with recent three dimensional imaging (Brelje *et al.*, 1989), have revealed a remarkable architecture and cellular organization of pancreatic islets ideal for rapid, yet finely controlled, responses to changes in blood glucose levels. It could not be predicted that such a structure could be produced *in vitro*, particularly when one considers that during
20 embryogenesis, islet development within the pancreas appears to be initiated from undifferentiated precursor cells associated primarily with the pancreatic ductal epithelium (Pictet *et al.*, 1972) i.e. non-islet cells. The ductal epithelium rapidly proliferates, then subsequently differentiates into the various islet-associated cell populations (Hellerstrom, 1984; Weir *et al.*, 1990; Teitelman *et al.*, 1993; Beattie *et al.*, 1994). The resulting islets are
25 organized into spheroid structures in which insulin-producing β cells form a core surrounded by a mantle of non- β cells. For the most part, glucagon-producing, α cells (if the islet is derived from the dorsal lobe) or alternatively, pancreatic peptide-producing PP cells (if the islet is derived from the ventral lobe), reside within the outer cortex (Brelje *et al.*, *supra*,
30 1989; Weir *et al.*, *supra*, 1990). Somatostatin-producing δ cells, which are dendritic in nature, reside within the inner cortex and extend pseudopodia to innervate the α (or PP) cells and the β cells. These spheroid islet structures tend to bud from the ductal epithelium and move short distances into the

5 surrounding exocrine tissue. Angiogenesis-induced vascularization results
in direct arteriolar blood flow to mature islets (Bonner-Weir *et al.* 1982;
Teitelman *et al.*, 1988; Menger *et al.*, 1994). Since blood glucose can
stimulate β cell proliferation, vascularization may act to increase further the
10 numbers of β cells. Similarly, neurogenesis leads to the innervation of the
islets with sympathetic, parasympathetic and peptidergic neurons (Weir *et al.*,
supra, 1990). That we have been able to produce functional islet-like
structures *in vitro* which can then be implanted to produce pancreas-like
structures, is therefore quite remarkable.

Unfortunately, the cellular organization of the islet can be destroyed
15 in diseases such as type I. insulin dependent diabetes (IDD), in which a
progressive humoral and cell-mediated autoimmune response results in
specific destruction of the insulin-producing β cells (Eisenbarth, 1986;
Leiter *et al.*, 1987). Because the β cell is considered to be, for the most
part, a differentiated end-stage cell, it is believed that the body has limited
20 capacity to generate new β cells, thus necessitating regular life-long insulin
therapy once the β -cell mass is destroyed. However, in experimental
animals, the β -cell mass has been shown to increase and decrease in order to
maintain euglycemia (Bonner-Weir *et al.*, 1994). The plasticity can occur
through two pathways of islet growth: first, by neogenesis, or growth of
25 new islets by differentiation of pancreatic ductal epithelium, and second, by
hypertrophy, or expansion through replication of preexisting β cells.
During embryogenesis, the β -cell mass initially expands from
differentiation of new cells, but by the late fetal stages the differentiated β
cells replicate. Replication, then, is likely to be the principal means of
30 expansion after birth, but the capacity to replicate appears to diminish with
age. Adult islet cells have been shown to replicate by responding to stimuli
known to initiate neonatal islet cell growth, e.g., glucose, growth hormone,
and several peptide growth factors (Svenne, 1992; Hellerstrom *et al.*, 1988;

5 Bonner-Weir *et al.*, 1989, Marynissen *et al.*, 1983; Neilsen *et al.*, 1992;
Brelje *et al.*, 1993). These observations suggest that the low level of β -cell
growth in the adult can accommodate functional demands. For example,
during pregnancy or chronic obesity, β cell mass increases significantly yet
is reversible since, following termination of pregnancy or after weight loss,
10 an increased β cell death via apoptosis quickly reduces β cell mass.

It is generally accepted that all pancreatic endocrine cell types
differentiate from the same ductal epithelium (Pictet *et al.*, 1972, *supra*;
Hellerstrom, 1984, *supra*; Weir *et al.*, 1990, *supra*; Teitelman *et al.*, 1993,
supra), but whether they are derived from a common stem/precursor cell is
15 uncertain. In normal adult pancreas, approximately 0.01% of the cells
within the ductal epithelium will express islet cell hormones and can be
stimulated to undergo morphogenic changes to form new islets, reminiscent
of neogenesis. This neogenesis has been induced experimentally by dietary
treatment with soybean trypsin inhibitors (Weaver *et al.*, 1985), high levels
20 of interferon- γ (Gu *et al.*, 1993), partial pancreatectomy (Bonner-Weir *et al.*, 1993),
wrapping of the head of the pancreas in cellophane (Rosenberg *et al.*, 1992),
specific growth factors (Otonkoski *et al.*, 1994) and the onset of
clinical IDD. Recently, attention has focused on the *Reg* gene (Watanabe *et al.*, 1994,
Otonkoski *et al.*, 1994), identified in a subtracted cDNA library of
25 regenerating rat islets, as a controlling element in the neogenesis of islet β
cells. Up-regulation of the *Reg* gene (e.g., by hepatocyte growth
factor/scatter factor) induces β cell proliferation resulting in increased mass,
while down-regulation of the *Reg* gene (e.g., by nicotinamide) induces
differentiation of the 'pre- β ' cells to mature cells. Thus, a population of
30 precursor/stem cells remain in the adult pancreatic ducts and differentiation
of this population can be evoked *in vivo* in response to specific stimuli.
This action may actually occur continuously at low levels.

5 Although intensive efforts have been made to reproduce islet neogenesis *in vitro*, minimal success has been achieved. We now describe, for the first time, conditions which permit the growth and expansion of mammalian-derived islet-producing stem cells (IPSCs) in culture, as well as their differentiation to islet-like structures.

10 Numerous strategies (e.g., bone marrow replacement, immunosuppressive drugs and autoantigen immunizations) have been investigated as possible means to arrest the immunological attack against the pancreatic β cells. However, for these approaches to be effective, individuals who will eventually develop clinical disease must be identified.

15 Most often, patients are identified too late for effective intervention therapy since the immunological attack has progressed to a point where a large percentage of the β cells have already been destroyed. Because the β cell is thought to be an end-stage differentiated cell, it was previously believed that the body has little capacity to regenerate new β cells, thus necessitating

20 regular life-long insulin therapy. Recently, one approach to overcome this problem has been islet cell transplantation. Islet cell transplantation has the disadvantage that the islets are allogeneic which, in turn, can invoke an allo-immune response. Thus, there would be major advantages to growing Islets of Langerhans containing functional β cells directly from IDD patients.

25

Brief Summary of the Invention

 The subject invention concerns the discovery that functional islets containing insulin-producing β cells, as well as other islet cell types, can be grown in long-term cultures from pluripotent stem cells, which give rise to

30 islet producing stem cells, IPSCs.

 The novel methods of the subject invention take advantage of the discovery that IPSCs exist even in the pancreas of adult individuals. The cells can be cultured in a minimal, high amino acid nutrient medium that is

5 supplemented with normal serum which is preferably derived from the same
mammalian species which serves as the origin of the islet cells (homologous
serum). Several discrete phases of cell growth result in selection of IPSCs
and subsequent progeny which are then induced to differentiate and form
islet-like structures which are distinguishable from pseudo-islet or pseudo-
10 pancreatic tissue of the prior art. In a first phase, primary culture of cells
from a pancreas are placed in a low serum, low glucose, high amino-acid
basal medium. This culture is then left undisturbed for several weeks to
permit establishment of stromal cells and to allow the vast majority of
differentiated cells to die. Once this stromal cell layer is mature, cell
15 differentiation can be initiated by re-feeding the cell culture with the high
amino acid medium supplemented with homologous normal serum plus
glucose. After an additional period of growth, functional islets containing
cells which produce insulin, glucagon, somatostatin and other endocrine
hormones can then be recovered using standard techniques.

20 It was not previously known or suspected that pancreatic-derived
non-islet cells (ductal derived cells) could be used to grow new islet-like
structures, including β cells, in culture. The fortuitous discovery of culture
techniques for growing islet-like tissue *in vitro* eliminates what had
previously been a substantial and longstanding barrier to diabetes research.
25 The novel methods and materials described herein enable a better
understanding of the mechanisms of diabetes. Furthermore, the ability to
produce islet-like structures from IPSCs in culture now makes certain
therapies for diabetes possible for the first time. For example, in
accordance with the subject invention, new cultured islets from diabetic
30 individuals can be implanted in a patient as a way to control or eliminate the
patient's need for insulin therapy because the cultured islets and/or islet cells
are able to produce insulin *in vivo*. Thus, the subject invention also
concerns the use of the *in vitro* grown islets of the subject invention for

5 implantation into a mammalian species for *in vivo* treatment of IDD.

 The subject invention also greatly facilitates genetic engineering of islet cells to resist subsequent immunological destruction. For example, the cultured islet cells can be transformed to express a protein or peptide which will inhibit or prevent the destructive immune process. Other useful
10 proteins or peptides may be expressed. In addition, expression of specific autoantigens, such as GAD, 64 kD islet cell surface antigens (see Payton *et al.* 1995), or any other markers identified on the differentiated pancreatic cells, can be eliminated by standard gene knock-out or selection procedures to produce differentiated pancreatic cells which are not or are less
15 susceptible to auto-immune attack. Methods for producing such mutant or knock out cell lines are well known in the art and include, for example, homologous recombination methods disclosed in U.S. Patent No. 5,286,632; U.S. Patent No. 5,320,962; U.S. Patent No. 5,342,761; and WO 90/11354; WO 92/03917; WO 93/04169; WO 95/17911, all of which are herein
20 incorporated by reference. In addition, a universal donor cell is produced by preparing a stem cell modified so as not to express human leukocyte antigen (HLA) markers as the cell differentiates into a pancreas-like structure (see especially WO 95/17911 *supra*).

 Thus, the ability to grow functioning islets *in vitro* from the
25 pancreatic cells of an individual represents a major technical breakthrough and facilitates the use of new strategies for treating and studying IDD. The discovery that pluripotent stem cells exist in adult pancreas circumvents (without excluding) the need to use fetal tissue as a source of cells.

 The subject invention also concerns the islet cells produced *in vitro*
30 according to the methods described herein. These cells are produced from a mammalian pancreatic cell suspension cultured *in vitro* and give rise to functional islet cells and islet-like tissue structures.

5 The subject invention further concerns the *in vitro* growth, propagation and differentiation of a pancreatic stem cell, i.e., a progenitor cell or cells that give rise to the formation of all of the different types of cells and tissue that make up a normal pancreas. Moreover, the subject invention concerns the *in vivo* use of *in vitro* grown pancreatic stem cells to
10 produce pancreas-like structures or an "ecto-pancreas" organ that exhibits functional, morphological and histological characteristics similar to those observed in a normal pancreas. Thus, the ability to produce a functional "ecto-pancreas" *in vivo* from *in vitro* grown pancreatic cells can be used to treat, reverse or cure a wide variety of pancreatic diseases that are known to
15 result in or from damage or destruction of the pancreas.

Brief Summary of the Figures

Figures 1A through 1D show cells grown according to the procedures of the subject invention.

20 **Figure 2** shows an islet like structure grown according to the subject invention.

Figure 3A through 3H shows sequential stages in the development of an islet-like structure *in vitro* from 3A, which shows a few cells after several weeks in culture, which have survived and which begin to "bud" (Figure 3B, dark structure in top righthand of field), and divide (Figure 3C
25 several locations in field), and to form highly organized structures (Figures 3D-3H) under the culture conditions described herein.

Figure 4 shows photomicrographs of the structures shown in Figures 3G-3H, showing the highly organized morphology thereof.

30 **Figure 5** shows H/E staining of an islet-like structure cross-sections showing the highly organized morphology of the structure with β -cells in the center and glucagon-producing cells at the periphery.

5 **Figure 6A through 6F** shows a series of micrographs in which an islet-like structure, such as that shown in Figure 3H, is harvested from a primary culture. In Figure 6B, the structure has disintegrated, and most of the cells have died, but in Figure 6C a new structure develops. In Figure 6D, several new structures have formed. This series of serial passage steps can be repeated a number of times until the IPSCs become depleted. In this event as the structure disintegrates, as in Figure 6E, instead of new structures being formed, the differentiated cells multiply, as shown in Figure 6F. It is this type of proliferated differentiated cell that is thought to have been produced by workers such as Coon *et al.* (see WO 94/23572, *supra*).

15 **Figure 7** shows data from control and implant NOD mice after cessation of insulin therapy.

Figure 8 shows an ecto pancreas.

20 Abbreviations and Definitions

 IPSCs are Islet Producing Stem Cells. IPSCs are a small population of cells derived from ductal epithelial cells (i.e., these cells are pancreas-derived but are not differentiated islet cells) discovered in fetal or adult pancreas which, according to this invention, have the capacity of giving rise to islet-like structures *in vitro*. When ductal epithelial cells are implanted *in vivo*, a pancreas-like structure is formed. When the pancreas-like structure and ductal epithelial cells are implanted in a location other than the natural pancreatic location *in vivo*, the pancreas-like structure is referred to as an ecto-pancreas.

30 Pluripotent pancreas stem cells are cells discovered in the pancreas which give rise to the IPSCs.

 Mature islet cells are differentiated cells which arise from IPSCs and which produce pancreatic hormones.

5 Islet-like structures, or young-islets, are highly-organized structures of cells which we have discovered arise in culture from IPSCs (see Figure 3H, Figures 4A and 4B, and cross-section shown in Figure 5). The structures "bud" from foci formed by individual IPSCs after most of the cells, which are not IPSCs, which are placed into culture from dissociated
10 pancreatic tissue, have died. Upon implantation of the islet-like structure, final differentiation occurs to produce fully mature islet cells.

Detailed Description of the Invention

15 According to the subject invention, functional Islets of Langerhans can for the first time be grown in *in vitro* cultures. The techniques of the subject invention result in cell cultures which can produce insulin, glucagon, somatostatin or other endocrine hormones. Other useful proteins may also be produced by, for example, transforming the islet cell with DNA which encodes proteins of interest. The ability to grow these functional cell
20 cultures enables those skilled in the art to carry out procedures which were not previously possible. In the following disclosure, the term islet-like structure should be read as being interchangeable with the term "young-islets", because these *in vitro* produced structures have most of the attributes of islets produced *in vivo* during normal neogenesis. The immature nature
25 of these structures permits implantation *in vivo* with rapid final differentiation and vascularization ensuing to provide a functioning replacement to damaged or otherwise compromised islets in recipients such as diabetic or prediabetic mammals, in need of such treatment.

30 The method of the subject invention involves making suspensions of cells, including stem cells, from the pancreas of a mammal. Preferably, the stem cells would be from the pancreas of a prediabetic mammal. However, it is also contemplated that islet producing stem cells, IPSCs, from mammals already showing clinical signs of diabetes, can be utilized with

5 the subject invention. The cell suspensions are prepared using standard techniques. The cell suspension is then cultured in a nutrient medium that facilitates the growth of the IPSCs, while at the same time severely compromising the sustained growth of the differentiated or mature cells other than IPSCs. In a preferred embodiment, the nutrient medium is one
10 which has a high concentration of amino acids. One such medium is known as Click's EHAA medium and is well known and readily available to those skilled in the art (Peck and Bach, 1973, herein incorporated by reference for this purpose). Other equivalent nutrient media could be prepared and utilized by those skilled in the art. What is required for such media is that
15 they have little or no glucose (less than about 1 mM) and low serum (less than about 0.5%). The high amino acid concentrations are preferably of amino acids known to be essential for the cells of the species being cultured, and provide a carbon source for the cultured cells. In addition, at least one rudimentary lipid precursor, preferably pyruvate, is provided. These
20 conditions are so stressful to most differentiated cells types that they do not survive. Surprisingly, however, upon extended culture of cells from pancreatic tissue without re-feeding (about 3 weeks) IPSCs do survive and after extended culture, begin to proliferate. Subsequent culture phases employ media supplemented with normal serum from the same species of
25 mammal from which the islet cells originate. Thus, in the case of mouse islets, the medium is supplemented with normal mouse serum, whereas in the case of human islet cells, the medium is supplemented with normal human serum. The preparation of normal serum is well known to those skilled in the art. The concentration of normal serum used with the cell
30 culture method of the subject invention can range from about 0.5% to about 10%, but for mice is preferably about 1%. For human serum, a higher concentration is preferred, for example, about 5%.

5 The cell suspension prepared in the nutrient medium supplemented with normal serum and about 2.5-10 mM glucose is then incubated under conditions that facilitate cell growth, preferably at about 35-40°C and, preferably, in an atmosphere of about 5% CO₂. This incubation period is, thus, carried out utilizing standard procedures well known to those skilled in the art. During this time, stromal or ductal epithelial cells proliferate and establish a monolayer which will ultimately give rise to islet-like structures. The initiation of cellular differentiation can be brought about by re-feeding the cultures with Click's EHAA or like medium supplemented with normal serum as discussed above. Rapid re-feeding was found to induce extensive islet foci and islet-like structure formation with considerable cell differentiation. We have found that cellular differentiation is further enhanced by inclusion of relatively high concentrations of glucose (about 10-25 mM and preferably 16.7 mM) in the re-feed medium. In addition, it is contemplated that any of a number of other biological factors, including, but not limited to, factors which up-regulate the Reg gene, such as hepatocyte growth/scatter factor, and other cellular growth factors, such as insulin-like-growth factor, epidermal growth factor, keratinocyte growth factor, fibroblast growth factor, nicotinamide, and other factors which modulate cellular growth and differentiation can be added to the cultures to optimize and control growth and differentiation of the IPSCs. By employing any of these various factors, or combinations thereof, at different stages, at different seeding densities and at different times from seeding in the course of IPSC differentiation, IPSC cultures are optimized. In addition, factors produced by the IPSC cultures in the course of differentiation which augment growth can be isolated, sequenced, cloned, produced in mass quantities, and added to IPSC cultures to facilitate growth and differentiation of those cultures. The relevant factors are identified by concentrating IPSC culture supernates from early, intermediate and late

5 stages of differentiation and testing for the ability of these concentrates to
augment IPSC growth and differentiation. Positive effects are correlated
with molecular constituents in the concentrates by two dimensional gel
electrophoresis of positive and negative supernates, purification and N-
terminal sequencing of spots present only in the positive concentrates and
10 subsequent cloning and expression of the genes encoding these factors.

Upon histological examination of the cells in the islet-like structures,
at least three distinct cell types were identifiable and appeared similar to
islet cells prepared from islets of control mice. The time required for cell
differentiation to occur within these foci decreased as the frequency of re-
15 feeding was increased.

We have been able to propagate and expand islet-producing cultures
through the serial transfer of islet-derived stromal cells plus islet foci to new
culture flasks. This facilitates generating sufficient numbers of islets as
required for use in methods described herein, for example, for reversing the
20 metabolic problems of IDD.

In order to determine whether the islet-like structures and/or islet
cells produced *in vitro* according to the subject inventions could reverse
IDD, the islet-like structures were implanted into NOD mice. Mice that
received the islet implants exhibited a reversal of insulin-dependent
25 diabetes, whereas untreated NOD mice showed signs of clinical disease. In
addition, no autoimmune pathogenesis was observed during the duration of
the implants. Thus, islet implants of the subject invention can be used *in*
vivo to treat diabetes in mammals, including humans.

In a preferred embodiment of the subject invention, the progression
30 of diabetes can be slowed or halted by re-implantation of autologous islets
engineered to be resistant to specific factors involved in the immunological
attack. For example, the islets can be engineered so that they are resistant to
cytotoxic T cells (see, for example, Durinovic *et al.*, 1994, identifying islet

5 specific T-cells and T-cell receptor sequences which are similar to insulinitis-inducing T-cells of diabetic mice; Elias and Cohen, 1994, identifying peptide sequences useful in diabetes therapy in NOD mice by turning-off production of specific diabetogenic T-cell clones: Conrad *et al.*, 1994, describing a membrane-bound, islet cells superantigen which triggers
10 proliferation of islet infiltrating T-cells; Santamaria *et al.*, 1994, describing the requirement of co-expression of B7-1 and TNF α for diabetes and islet cell destruction; any of these antigens may be eliminated according to known methods to improve the resistance of the implanted islets against immunologic attack). The availability of long-term cultures of whole islets
15 can also be used in investigations into the pathogenesis of IDD, including the cellular recognition of β cells, the mode of islet infiltration, and the immune mechanisms of β cell destruction. Furthermore, this technology facilitates islet transplantation, autologous islet replacement, and development of artificial islets. The growth of these cells and islet-like
20 structures according to the procedures of the subject invention has great utility in teaching students and in increasing the understanding of important aspects relating to cell differentiation and function.

In a further embodiment of the subject invention, pluripotent pancreatic stem cells, which give rise to ISPCs, have been grown *in vitro*
25 from pancreas cells isolated from a mammal. A surprising discovery using these *in vitro* grown cells in conjunction with the methods of the subject invention, was the ability to grow and produce, *in vivo*, an organ that exhibited functional, morphological and histological features and characteristics similar to a normal pancreas, including cell differentiation, to
30 form endocrine and exocrine tissues. The ecto-pancreas, (a pancreas-like organ situated at an abnormal site within the body cavity), produced *in vivo* according to the subject invention, represents a major scientific discovery and provides a novel means for studying, treating, reversing or curing a

5 number of pancreas-associated pathogenic conditions including, but not limited to, pancreatitis, pancreatic cancer and IDD. This is accomplished by removal of the diseased tissue and implantation of the islet-like structures produced according to this invention. In addition, the islet-like structures can be implanted into the natural pancreatic site.

10 Because this invention provides a method for culturing pancreatic stem cells and production of young islets *in vitro*, study of the growth and differentiation of this cell-type is now possible. Accordingly, all of the known methods of cell culture, purification, isolation and analysis can be brought to bear on the significant questions regarding how many types of
15 cells are involved in pancreatic cell differentiation. These methods include, but are not limited to, fluorescence activated cell sorting (FACS), magnetic bead usage (as in, for example, the use of the commercially available DYNA BEADS which are specifically adapted for this purpose), use of magnetically stabilized fluidized beds (MSFB, see U.S. Patent No.
20 5,409,813), and any of a number of other methods known in the art. The pathway for this process is now amenable to dissection. Identification of markers (including cell-surface, intracellular, protein or mRNA), specific to every stage of this process, are also now readily identifiable through application of standard techniques including, but not limited to: production
25 of antibodies, including monoclonal antibodies, to cells, cell surface markers, and cellular components which differ throughout the process of pancreatic stem cell maturation; production of T-lymphocytes which specifically respond to antigens expressed by the pancreatic cells at different stages in the maturation and differentiation process (see, for
30 example, Wegmann *et al.*, 1993); identification and elimination of cell surface markers recognized by T-cells and which, therefore, result in differentiated β -cell destruction if present (see references above); identification of factors significant in bringing about the different stages of

5 maturation and the different factors produced by the differentiating cells:
subtractive hybridization of nucleic acids isolated from cells at different
stages in the maturation process. enabling pinpointing of gene products
significant to each aspect of the cellular differentiation; differentiated
display PCR (see, Liang *et al.*, 1992); arbitrarily primed PCR (see, Welsh *et*
10 *al.*, 1992); representational difference analysis PCR (RDA-PCR) (see,
Lisitsyn, 1993); encapsulation of single pancreatic progenitor cells or
populations thereof for implantation in appropriate host organisms, thereby
providing advantages that such methods have demonstrated in implantation
of other types of progenitor or engineered cells (see, Altman *et al.*, 1994);
15 genetic engineering of the pancreatic progenitor cells to produce cells less
susceptible to autoimmune attack, such as by knock-out of autoantigen
genes, or insertion of resistance enhancing genes; other genes which may be
inserted into the IPSCs include those which provide altered cellular surface
antigens or which provide different biochemical properties to the internal
20 milieu of the cells; these include genes which express enzymes which
increase or decrease the sensitivity of the cells to glucose or genes which
increase or decrease the responsiveness of the cells to growth factors; in
addition, genes which increase or decrease the production of insulin,
glucagon or somatostatin may also be introduced; examples of how these
25 types of modifications can be introduced into the IPSCs include
electroporation, virus vectors, transfection or any of a number of other
methods well known in the art (see, for example, WO 95/17911; WO
93/04169; WO 92/03917; WO 90/11354; U.S. Patent No. 5,286,632; WO
93/22443; WO 94/12650; or WO 93/09222; all of which are incorporated by
30 reference for this purpose); production of universal donor (knock-out) cells
which, for example, have deleted or otherwise modified human leukocyte
antigens (see, WO 95/17911, *supra*). Because this process does not depend
on the use of fetal tissue, it is possible to remove pancreatic tissue from a

5 mammal suffering from IDD or at risk of suffering from IDD, grow an islet-like structure *in vivo* and re-implant that structure into the individual to produce physiologically relevant amounts of insulin in response to fluctuations in blood glucose.

10 In view of the foregoing disclosure and the exemplary support which follows, it should be recognized that the scope of the appended claims extends to the various embodiments and aspects of this invention which those skilled in the art will recognize are enabled by this significant invention.

15 It will also be recognized that data presented herein reveal that *in vitro* neogenesis of islets from isolated pluripotent stem/progenitor cells is possible, but involves several distinct phases of growth, including: 1) Establishment of a stromal, or "nurse", cell monolayer of ductal epithelial cells which permits the generation of IPSCs; 2) Induction of stem/progenitor cell proliferation with specific culture conditions which
20 promote cyclical regeneration of IPSCs and also prevent premature differentiation of the IPSC; 3) Expansion and differentiation of the α , β and δ cells. This step is dictated by the culture environment, as differences in culture nutrients and growth factors result in islets containing different percentages of the various islet cell types. Identification of *in vitro*
25 conditions which induce the β cell to its final maturation stage, i.e., formation of insulin-containing granules and glucose responsiveness can also now be achieved. A factor present *in vivo* which achieves this final differentiation is identified by addition of cellular extracts or growth factors to the IPSC cultures.

30 We have maintained primary IPSC cultures for up to 10 months and secondary cultures an additional 14-16 months each capable of expansion and differentiation to form islet-like structures. While the ability to grow functioning islets from prediabetic adults represents a major technical

5 breakthrough and focuses attention on possible new strategies for attaining a
cure for IDD, perhaps the most important aspect of this work is the
demonstration that pluripotent stem/progenitor cells, which can give rise to
IPSCs and pancreas-like structures when implanted, exist in the islets of
both normal and (pre)diabetic adults. This finding will eliminate the need to
10 use either fetal, allogenic or xenogeneic tissue for transplantation of β cells
into IDD patients; to develop novel strategies to reverse hypoglycemia *in*
vivo; study immunological responses to newly implanted islets; and/or
create islets resistant to immunological attack.

It is tempting to speculate, based on the data presented herein, that
15 the well-documented period of remission in type I IDD patients following
onset of disease might actually represent a time when stem cell growth is
induced, only to be subsequently overwhelmed by the on-going
autoimmune reaction. Since re-implantation of autologous islets is thought
to require cells engineered to be resistant to the immunological attack,
20 identification and culture of islet stem cells as disclosed herein is essential
for the genetic engineering efforts described above.

Surprisingly, the *in vitro*-generated islet implants of this invention
showed no signs of immunological attack over the time period studied here.
It is possible that the autoantigen(s) are not expressed on cultured cells, or
25 that the autoantigen(s) cannot be presented since culture dilutes out the
macrophages, or such implants may induce peripheral tolerance. The
availability of long-term cultures of whole islets facilitates investigations
into the pathogenesis of IDD, including the cellular recognition of β cells,
the mode of islet infiltration, and the immune mechanisms of β cell
30 destruction. Furthermore, this technology facilitates islet transplantation,
autologous islet replacement, development of artificial islets and reduces the
need for insulin therapy.

5 Accordingly, this invention provides a method for the *in vitro* growth of islet producing stem cells, IPSCs, to produce an islet-like structure. The method comprises culturing pancreatic cells from a mammalian species in a basal nutrient medium supplemented with normal serum at below about 0.5% and glucose at below about 1 mM, allowing the IPSCs to grow for at
10 least about 3 weeks, and initiating cellular differentiation into mature islet cells by re-feeding the IPSCs in culture with a nutrient medium supplemented with normal serum at about 0.5-10% and glucose at about 2.5 mM-10 mM. The pancreatic cells may be from any mammal, including humans and mice, and the serum is from the same species. The medium
15 preferably contains all of the amino-acids essential to growth of cells from the species being cultured and in such quantity as to ensure that the culture does not become depleted. Upon re-feeding, the re-feed medium preferably contains glucose and serum in sufficient quantities to stimulate differentiation. Furthermore, according to this method, once differentiation
20 has begun, the cells are preferably re-fed frequently (about once per week). This method produces islet cells and islet-like tissue structures.

 This method also provides a source of endocrine hormones, including, but not limited to, insulin, glucagon and somatostatin, which can be recovered from the IPSC culture medium or which can be directly
25 released into a mammal by implantation of the islet-like structures into the tissue of a mammal to produce a pancreas-like structure. Such implantation provides a method for treating pancreatic disease in a mammal by implanting an islet-like structure to produce a pancreas-like organ in the mammal. In one embodiment, the islet cells or islet-like structure of this
30 invention is genetically modified so as to not produce IDD autoantigens or HLA markers such that it does not express insulin dependent diabetes associated autoantigens, other than insulin, or which has been modified so that it does not express human leukocyte antigens, as said stem cell

5 differentiates into said pancreas-like organ. Furthermore, the pancreatic stem cell may be encapsulated in an insulin, glucagon, somatostatin and other pancreas produced factor permeable capsule. Also provided is a method for analyzing the differentiation of pancreatic stem cells which comprises culturing at least one pancreatic stem cell *in vivo*, and inducing
10 said at least one stem to begin differentiation into a pancreas-like structure. This method also permits identification of mRNA or protein markers specific to a plurality of different stages in the differentiation process. The protein markers may be expressed on the cell-surface, be secreted, or they may be intracellular. In another aspect of this invention a ligand binding
15 molecule and a method for making a ligand-binding molecule which selectively binds to pancreatic stem cells or to more differentiated pancreatic cells is provided. The method comprises contacting a naive B-lymphocyte or T-lymphocyte with an identified protein marker, and culturing and expanding the B-lymphocyte or T-lymphocyte to obtain a
20 population of cells which produce the ligand-binding molecule. These ligand-binding molecules thus provide a method of isolating a pancreatic stem cell or partially differentiated pancreatic cells at any stage between that of a pancreatic stem cell and a fully differentiated pancreatic cell. This method comprises selecting the cell from a population of cells comprising
25 the cell, which a specific ligand-binding molecule which binds to a protein marker expressed by the cell at a given state of differentiation. Alternatively, the method comprises selecting and removing other cells from a population of cells comprising the cell with a specific ligand binding molecule which binds to a protein marker absent on the surface of the cell.
30 In yet another aspect, this invention provides a method for treating a mammal suffering from, or at risk of IDD, which comprises:

- a. removing pancreatic tissue from the mammal;

5 b. culturing pluripotent pancreatic cells present in the pancreatic tissue *in vitro* to generate islet-like structures; and

 c. implanting said islet-like structures into said mammal.

 In a further aspect of this invention, there is provided an IPSC modified so as not to express insulin dependent diabetes autoantigens in
10 either the undifferentiated or in the differentiated state of the IPSC. Preferably, the autoantigen which is not expressed as a result of the modification is selected from GAD, 64 kD islet cell antigen, and HLA markers.

 As part of the method of this invention, a method for *in vitro*
15 neogenesis of islets from pluripotent stem or progenitor cells is provided with comprises:

 a. establishing a stromal, or "nurse", cell monolayer of ductal pancreatic epithelial cells which permits the generation of IPSCs;

 b. inducing stem/progenitor cell proliferation with culture
20 conditions which promote cyclical regeneration of IPSCs and also prevent premature differentiation of the IPSC; and

 c. expanding and differentiating the IPSCs to produce an islet-like structure comprising α , β , and δ cells. Preferably, the culture-generated islet-like structure is characterized by large, differentiated cells which stain
25 with insulin-specific stain in the center of the islet-like structure; small differentiated cells which stain with glucagon-specific stain at the periphery; and proliferating and undifferentiated cells which do not stain with any of the endocrine hormone-specific stains in the inner cortex. The structure is further characterized in that, upon breaking the structure into single cell
30 suspensions by mechanical or other means in the presence of a proteolytic enzyme and subsequent staining of individual cells, individual cell populations which stain either with glucagon-specific stain (α cells), insulin-specific stain (β cells) or somatostatin-specific stain (δ cells) are

5 observed. The method of *in vitro* neogenesis of islets according to this invention preferably comprises:

- a. dispersing and leaving undisturbed pancreatic cells in a minimal culture medium comprising little or no glucose, serum at a concentration below about 0.5%, essential amino acids for the cells of the species from which the pancreatic cells were obtained, and a lipid source, until about 99% of the cells in said culture have died (Phase I);
- b. re-feeding the culture of step (a) with the minimal medium supplemented with about 1-10 mM glucose and about 0.5%-10% serum (but less than a toxic amount) and re-feeding about once a week until rapid proliferation occurs;
- c. re-feeding the culture of step (b) with the minimal medium supplemented with 0.5%-10% serum and about 10-25 mM glucose and, optionally, added growth or cellular factors (Phase III);
- d. allowing islet-like structures to bud into the medium;
- e. recovering the islet-like structure.

This process may be repeated several times by serially transferring epithelial cells plus early-stage, proliferating islet-like structures in culture *in vitro*.

25 As used herein, the term "growth" refers to the maintenance of the cells in a living state, and may include, but is not limited to, the propagation and/or differentiation of the cells. The term "propagation" refers to an increase in the number of cells present in a culture as a result of cell division.

30 Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

5 Example 1 - Culturing of Functional Islets of Langerhans

Single cell suspensions of islet cells were prepared from whole islets isolated from the pancreas of 19-20 week old prediabetic male NOD/UF mice, as detailed elsewhere (Shieh *et al.*, 1993). Typically, about 25% of the male mice in a NOD colony will have overt IDD at this age and will have severe insulinitis. The islet cells were re-suspended in glucose depleted or glucose-free Click's EHAA medium (Peck and Bach, 1973, *supra*; Peck and Click, 1973) supplemented with normal mouse serum (NMS) to 0.25%, plated in a 25 cm² tissue culture flask, and incubated at 37°C in a 5% CO₂ atmosphere. At this stage, two outcomes are possible: first, the islet-infiltrating cells may dominate, thus permitting the establishment of immune cell lines, or second, ductal epithelial cells (often referred to a stromal cells in these cultures) may dominate, thus allowing the growth of a "nurse cell" monolayer. Growth of stromal-like cell monolayers appeared to result when islet-infiltrating cells were plated simultaneously but in limited numbers. Enrichment of the islet cells with decreased numbers of infiltrating cells can be achieved by gradient separation (Jarpe *et al.*, 1991, *supra*). The vast majority (>99%) of the original cells die during this incubation period, leaving a small number of epithelial-like cells attached to the culture dish (Figures 1A and 3A, Stage I). Stromal cell cultures, when left undisturbed for 4-5 weeks (i.e., no re-feeding) proliferated to cover the entire bottom surface of the culture vessel (Figures 3C and 3D).

Differentiation and endocrine hormone expression of the cultures was initiated by re-feeding the cultures with Click's EHAA medium supplemented with NMS and a sugar solution comprising glucose or sucrose or other sugar equivalents. Typically, the sugar is glucose. The concentration of glucose can be between about 0.25 mM to about 10 mM, but typically is about 2.5 mM. *Normal NOD or NMS serum at about 0.5% is also preferably included.* Techniques for re-feeding cell cultures *in vitro*

5 are well known in the art and typically involve removing from about 50% to about 90% of the old nutrient medium and adding fresh medium to the culture flask. Rapid re-feeding induced the formation of increasing numbers of centers of islet growth (referred to herein as foci) exhibiting cell differentiation. The rate of re-feeding can be, for example, at about one
10 week intervals. Preferably, the rate of re-feeding is at about 5 to 6 day intervals. Small rounded cells appeared on top of the epithelial monolayers, almost as if by budding (Figures 1B and 3D, Stage II).

At peak production, as many as 50-100 foci occurred simultaneously in a single 25 cm² (4 in²) tissue culture flask. Each individual rounded cell
15 underwent rapid proliferation, with the daughter cells forming cell clusters (Figure 1C). Rapid re-feeding induced increasing numbers of cell clusters as well as increased numbers of cells within each cluster. Induction of islet-like structures (Stage III) was enhanced through re-feeding of cultures with EHAA medium supplemented with normal mouse serum (0.5%) and high
20 levels of glucose (10 mM-25 mM and preferably about 16.7 mM glucose - See Figure 1D and 3E-3F). As the cell proliferation and differentiation proceeded, the organization of the islet took place and the islet even appeared to surround itself with a capsular material. Mature islets (Stage IV) appeared as smooth spheroids composed of tightly clustered cells
25 (Figure 3F-3H). This differentiation appears to be enhanced when serum from NOD mice is used rather than serum from other strains of mice, and higher levels of insulin-like growth factor (IGF), epidermal growth factor (EGF) and/or hepatocyte growth factor (HGF) in the NOD mouse serum are believed to be responsible for this effect. The islets generally grew to a
30 constant size (about 100-150 μ , Figure 2, although fusion of two clusters resulted in islets about twice the general size), then detached off of the stromal layers to float in the medium. these free-floating islets tended to break down within 48-72 hours, similar to what is observed when pancreatic

5 islets are isolated from *in vivo* sources and then cultured under similar conditions. Serial rounds of this process may then be conducted (see Figure 6A-6D and Example 5 below).

The islet-like structures, collected after natural detachment or removal from the stromal layers using a Pasteur pipette, were gently washed
10 in medium, then broken into single cell suspensions by reflux pipetting. Single cell suspensions were prepared by cytocentrifugation, then stained for general morphology and insulin production. The foci contained cells producing the endocrine hormones glucagon (α cells), insulin (β cells) and/or somatostatin (δ cells). Furthermore, the major population of cells
15 stained positive with anti-insulin antibody, indicating the major cell type contained in the cultured islet is an insulin-producing β cell. Figures 1A through 1D show the various cell types which develop during the culture process. Figure 2 shows a well-developed islet obtained after the *in vitro* culture of cells according to the method of the subject invention.

20 Example 2 - Culturing of Human Islet Cells

For culturing human islet cells, a procedure similar to that described in Example 1 was utilized. The procedure of the subject invention is particularly advantageous because it is not necessary to utilize fetal cells to
25 initiate the cell culture. In a preferred embodiment, the human cells can be suspended in Click's EHAA medium (or the equivalent thereof) supplemented with normal human serum. Preferably, the concentration of normal human serum used in the medium is about 0.25% -1% in phases I and II, respectively, and 5% during subsequent phases. The cultures should
30 be left undisturbed with no re-feeding, preferably for several weeks (Phase I). After about 4-5 weeks in culture, cell differentiation can be initiated by re-feeding the cultures with Click's EHAA medium supplemented with normal human serum and glucose as described in Example 1. Islet-like

5 structures can subsequently be collected and single cell suspensions prepared for further propagation as described in Example 1.

Example 3 - Implantation of *In Vitro* Grown Islet Cells

10 To test the efficacy of these *in vitro* generated islet-like structures to reverse the complications of IDD, approximately 150-200 foci plus some stromal cells grown *in vitro* according to the method of the subject invention from pancreatic tissue of NOD mice were dislodged from the tissue culture flask by reflux pipetting. The cells were then implanted beneath the kidney capsule of syngeneic diabetic NOD mice maintained by
15 daily insulin injections. Implantation was accomplished by puncturing the kidney capsule with a hypodermic needle, threading a thin capillary tube through the puncture site into the kidney, and injecting the islet foci directly into the cortex region. The capillary tube was carefully withdrawn and the puncture site cauterized. The surgical incision of each implanted mouse
20 was clamped until the skin showed signs of healing. The implanted mice were maintained on insulin injections for 4 days at the full daily dosage, and then for 2 days at the half daily dosage, after which the mice were completely weaned from further insulin treatment. Control animals consisted of diabetic NOD mice that did not receive an implant.

25 Within 8-14 days after weaning from insulin, control NOD mice showed a rapid onset of overt disease, including lethargy, dyspnea, weight loss, increased blood glucose levels (400-800 mg/dl), wasting syndrome, failure of wound healing and death within 18-28 days (Figure 7). Implanted NOD mice maintained a blood glucose level of about 180-220 mg/dl (which
30 is slightly above the normal range for mice), showed increased activity, rapid healing of surgical and blood-draw sites, did not develop dyspnea, and remained healthy until killed 55 days post-implant for histological studies (Figure 7). Similar observations have been seen with intra-splenic implants.

5 These data are consistent with the concept that the implanted *in vivo*-generated islets provide the necessary insulin to maintain stable blood glucose levels over the time course of the experiment.

Example 4 - *In Vivo* Production of Ecto-Pancreas

10 Histological examinations of the implant sites in mice that were implanted with the islet cells as described in Example 3 revealed an additional characteristic of the *in vitro* generated islet-forming stem cells. Implanted cells which "leaked" from the implant site of the kidney underwent additional proliferation and differentiation and formed a highly
15 structured ecto-pancreas. At first, the ecto-pancreatic tissue consisted entirely of proliferating exocrine cells which organized into an exocrine pancreas complete with innervating blood vessels. This exocrine pancreas progressed to form islet-like endocrine structures (see Figure 8). Thus, the *in vitro* cell cultures produced according to the methods of the subject
20 invention contain pluripotent pancreatic stem cells capable of regenerating a completely new pancreas. The growth of a pancreas containing both exocrine and endocrine tissue provides new methods for treatment of pancreatic diseases, including pancreatitis and pancreatic cancer.

Example 5 - Long Term Propagation of IPSCs

25 Long term propagation (> 1 year) of the IPSCs was achieved through serial transfers of small numbers of the epithelial cells plus a few early-stage, proliferating islet-like structures to new culture flasks. Cells from a single 25 cm² tissue culture flask have been expanded successfully to 5-10
30 150 cm² tissue culture flasks. Interestingly, serial transfer uniformly resulted in the islet structures "melting" away, similar to the detached islet-like structures, while new stromal monolayers formed (Figure 6A-6B). However, serially transferred cultures produced new islets far sooner than

5 primary cultures and in higher number (as many as 200-250 structures per square inch of culture-figures 6C-6D). However, eventually, after many rounds of serial growth and production of islet-like structures, a point is generally reached where after the islet-like structure "melts", only differentiated cells proliferate (see Figures 6E-6F). The same thing can
10 occur, in the absence of observable islet-like structure formation, if primary pancreatic tissue is grown in primary culture under conditions which do not kill most of the differentiated cells.

Example 6 - Analysis of Islet-Like Structures

15 Photomicrographics of serial sections of immature, culture-generated islet-like structures and sections thereof (shown in Figures 4 and 5, respectively) again demonstrate the uniformity of growth. Large, somewhat differentiated cells which stain weakly with insulin are observed in the islet center. Small differentiated cells which stained with glucagon were
20 apparent at the periphery, while a significant number of immature, proliferating, and undifferentiated cells which did not stain with any of the endocrine hormone antibodies were present in the inner cortex. To determine more precisely the cell phenotypes present within the *in vitro* grown islets, the islet-like structures were collected following detachment
25 from the epithelial monolayers, gently washed in medium, then broken into single cell suspensions by mechanical means, such as reflux pipetting in the presence of a proteolytic enzyme such as 0.25% trypsin. Slides of single cell suspensions were prepared by cytocentrifugation and stained for general morphology or cellular content. Several morphologically distinct mature
30 and immature cell types are observed following H/E staining. Furthermore, individual cell populations stained with either anti-glucagon (α cells), anti-insulin (β cells) or anti-somatostatin (δ cells) antibodies, indicating the pluripotent nature of the stem/progenitor cells giving rise to the islet-like

5 structures. These observations emphasize two points: first, the weak staining for endocrine hormones suggests the cells of *in vitro*-generated islets remain relatively immature, and therefore capable of further differentiation upon *in vivo* implantation, and second, the fact that > 100% of the cells could be accounted for by endocrine hormone staining indicates
10 that some cells must express both glucagon and insulin simultaneously, as reported recently by Teitelman *et al.* (Teitelman *et al.*, 1993, *supra*).

Example 7 - Limiting Dilution of Pancreatic Cells - Cloning of Single Pancreatic Stem Cells

15 According to the methods described above, pancreatic tissue is dispersed in a culture medium. To isolate single stem cells for clonal production of differentiated pancreatic cells, the dispersed pancreatic cells are subjected to limit dilution according to methods well known in the art. Thus, for example, serial ten-fold dilutions are conducted after an initial
20 evaluation of the number of cells/mL in the dispersed sample, such that the final dilution yields, at the most, an average of 0.3 cells per microtiter well or other container suitable for this type of dilution experiment. Thereafter, the cells are allowed to remain undisturbed until foci begin to develop. These foci are stem cells which have arisen from a single pluripotent stem
25 cell or IPSC and which can each be cultured to yield an islet-like structure for implantation to form a pancreas-like structure.

Example 8 - Identification of Markers Associated with Different Stages of Pancreatic Stem Cell Differentiation, and Production of Ligand Binding Molecules Specific to Each Stage of Differentiation

30 Foci of isolated stem cells produced according to Example 7 or by an analogous method are analyzed both prior to and after induction of differentiation according to Example 1 or by a similar method. The cells at

5 each stage, from stem cell to fully committed differentiated pancreatic cells.
are analyzed as follows:

A. Nucleic Acid: At each stage of differentiation, including
the undifferentiated progenitor cells and the fully differentiated pancreatic
cells, mRNA is isolated. This RNA is used to make cDNA according to
10 standard methods known in the art (Maniatis *et al.*, 1982) including, but not
limited to, PCR dependent amplification methods using universal primers,
such as poly A. Each amplification represents a library of message
expressed at each stage of pancreatic stem cell development. Accordingly,
message not present in stem cells but present in fully differentiated
15 pancreatic cells is identified by hybridizing the cDNA from each stage and
isolating message that remains unhybridized. Likewise, methods such as
differentiated display PCR, arbitrarily primed PCR or RDA-PCR (see
above) may be used. In this manner, message unique to each stage is
identified by subtraction of message present at other stages of
20 differentiation. Also, by this method, gene products at each stage of the
differentiation process are identified by expressing the product encoded by
the subtracted message. Antibodies, including monoclonal antibodies, are
then produced by using these gene products as antigens according to
methods well known in the art (see, Goding, J.W., 1986). These antibodies
25 are subsequently used to isolate cells from any given stage of differentiation
based on affinity for markers expressed on the cell surface of the pancreatic
cell. In addition, identification of specific markers which are expressed on
the surface of the differentiated pancreatic cells allows production of knock-
out lines of pancreatic cells by site-directed mutagenesis using the identified
30 sequences to direct mutations in stem cells according to methods such as
those disclosed in U.S. Patent No. 5,286,632, *supra*; U.S. Patent No.
5,320,962; U.S. Patent No. 5,342,761; and in WO 90/11354; WO 92/03917;
WO 93/04169; and WO 95/17911. Selection of mutant cells which do not

5 produce the knocked-out gene product is accomplished using the antibodies to the specific gene product selected against to provide clones of cells in which that product is absent.

10 B. Protein Markers: At each stage of differentiation, including the undifferentiated progenitor cells and the fully differentiated pancreatic cells, antibodies are generated to whole cells and subcellular fractions, according to standard methods known in the art. As specific examples of this process:

15 a) Production of rat anti-mouse IPSC mAbs: To enhance selection of B lymphocytes activated against IPSC-specific antigens, rats are immunized with normal mouse tissue followed by treatment with cyclophosphamide on day 7 post-immunization. Cyclophosphamide selectively kills the reactive B cells, leaving the rats unresponsive to normal mouse antigens. On day 14 post-immunization, the rats are re-challenged with cells collected from various stages of mouse IPSC cultures. Three to 20 four weeks after this secondary challenge, the rats are re-immunized with IPSC culture cells for three days, then fused with the SPO/2 myeloma partner. Positively reacting antibodies are selected and cloned.

25 b) Production of Mouse Anti-Human IPSC mAbs: Mouse anti-human IPSC mAbs are prepared using the same procedure as described above for the production of rat anti-mouse mAbs, except that mice are immunized with normal human tissue and then re-challenged after cyclophosphamide treatment with cells from various stages of human IPSC cultures.

30 c). Use of Anti-IPSC mAbs in the Identification of Various Differentiation Stages of Islet Cell Growth: The mAbs raised against IPSC cultured cells are used to sort by FACS or any other means known in the art, such as in magnetically stabilized fluidized beds (see below), the various cell populations defined by these reagents. Sorted cell populations

5 are examined for their stages of differentiation (e.g., co-expression of insulin, glucagon, somatostatin, β -galactosidase, tyrosine hydroxylase, the *Reg*-gene to name a few) and their growth capacity (e.g., their ability to initiate IPSC cultures).

10 Reagents which define cell surface and differentiation marks of cells involved in the neogenesis of islets are useful for the scientific community in this area of research. In addition, such reagents greatly facilitate the isolation (or enrichment) of IPSCs *per se*. Isolation of IPSCs permits testing of the efficacy of re-implanting IPSCs instead of whole islets into
15 IDD patients, or even implantation directly into the pancreas, circumventing the need for extra-pancreatic implants.

In addition, these antibodies are used to isolate cells from any given stage of differentiation based on affinity for markers expressed on the cell surface of the pancreatic cell. Identification of specific markers which are expressed on the surface of the differentiated pancreatic cells allows
20 production of knock-out lines of pancreatic cells. Cells which do not produce the undesirable gene product are selected by using the antibodies to select for clones of cells in which that product is absent. In an analogous fashion, markers significant to T-cell recognition and destruction of differentiated pancreatic cells are identified by activating naive T-cells with
25 whole pancreatic cells or subcellular fractions thereof, across the differentiation process. Identification of markers significant to T-cell activation allows subsequent modification of the stem cells to eliminate these marks and thereby produce cells which, in the differentiated state, are resistant to autoimmune destruction.

30
Example 9 - Isolation of Pancreatic Cells at Different Stages of Differentiation

5 Using the markers and ligand-binding molecules identified according to Example 8, pancreatic stem cells or partially or completely differentiated pancreatic cells can be isolated according to methods well known in the art. Accordingly, the methods for hematopoietic stem cell isolation disclosed in U.S. Patent No. 5,061,620; 5,437,994; 5,399,493; in which populations of
10 pure stem cells are isolated using antibodies to stem cell markers, are hereby incorporated by reference as if fully set forth herein. Likewise, the methods for mammalian cell separation from mixtures of cells using magnetically stabilized fluidized beds (MSFB), disclosed in U.S.
Patent No. 5,409,813, are hereby incorporated by reference as if fully
15 set forth herein. Antibodies to markers identified at each stage of pancreatic stem cell differentiation are attached to magnetizable beads, and cells are passed through the magnetically stabilized fluidized bed. Cells which adhere to the antibody bound magnetizable beads, or cells which flow through the bed, are isolated.

20 Any of a number of other methods known in the art for isolation of specific cells may be used for this purpose. These methods include, but are not limited to, complement destruction of unwanted cells; cellular panning; immunoaffinity chromatography, elutriation; and soft agar isolation techniques (see, Freshrey, R.I., 1988).

25

Example 10 - Analysis of Factors Which Trigger Pancreatic Stem Cell
Differentiation and Factors Produced at Different Stages of Stem Cell
Differentiation

30 Cells isolated according to the methods of Example 9 or like methods are cultured according to the method of Example 1 or like culturing method. Factors significant in inducing differentiation are assayed by adding different factors to the growth medium and observing the differentiation inducing effect on the cells. Thus, conditioned culture media from various

5 cells can be tested, and factors which cause pancreatic stem cell differentiation are isolated using induction of differentiation as a purification assay. Other factors such as glucose, other chemicals, hormones and serum fractions are similarly tested to isolate the significant differentiation inducing factors.

10 Factors produced at different stages of differentiation are isolated and analyzed from the conditioned culture medium of cells at each stage of the differentiation process. These factors are likewise tested for their autocrine effect on stem cells and further differentiation of partially differentiated stem cells.

15 Example 11 - Genetic Modification of Pancreatic Stem Cells to Produce Autoantibody, CTL Resistant, and HLA Modified Differentiated Pancreatic Cells

20 Pancreatic stem cells cultured according to Example 1 or 2 or isolated according to Example 8 are subjected to genetic modification according to any method known in the art to produce autoantibody and CTL resistant stem and differentiated pancreatic cells, according to methods such as those disclosed in U.S. Patent No. 5,286,632, *supra*; U.S. Patent No. 5,320,962, *supra*; U.S. Patent No. 5,342,761, *supra*; and in WO 90/11354, *supra*; WO 92/03917, *supra*; WO 93/04169, *supra*; and WO 95/17911, *supra*.
25 Alternatively, selection of resistant stem cells is accomplished by culturing these cells in the presence of autoantibody or IDD associated CTLs or CTLs activated with IDD specific autoantigens. As a result of these techniques, cells having increased resistance to destruction by
30 antibody or T-lymphocyte dependent mechanisms are generated. Such cells are implanted into an appropriate host in an appropriate tissue as disclosed above in Examples 3 and 4 to provide a pancreas-like structure which has increased resistance to destruction by autoimmune processes.

5 Likewise, the human leukocyte antigen profile of the pancreatic stem cell and differentiated cell is modified, optionally by an iterative process, in which the stem cell is exposed to normal, allogeneic lymphocytes, and surviving cells selected. Alternatively, a site directed mutagenesis approach is used to eliminate the HLA markers from the surface of the stem or
10 differentiated cells, and new stem cells thereby generated or isolated from pancreas-like structures are used to implant into a recipient mammal in need of such implantation.

 In a specific example, the adeno-associated virus (AAV) vector system carrying the neomycin-resistance gen, *neo* is used. AAV can be
15 used to transfect eukaryotic cells (Laface, 1988). In addition, the pBABE-bleo shuttle vector system carrying the phleomycin-resistance gene is used (Morgenstein, 1990). This shuttle vector can be used to transform human cells with bacteria-derived genes.

 a) Transfection of IPSCs: Cultured IPSCs are transfected
20 with either the retroviral segment of the pBABE-2-bleo vector by electroporation or the AAV-neo vector by direct infection. Adherent cells from established IPSC cultures are removed gently from the tissue culture flasks using C-PEG buffer (phosphate buffered saline supplemented with EDTA and high glucose). These cells are suspended in DMEM and 10%
25 fetal rat serum containing the retroviral stock, and in the case of pBABE-bleo, subjected to electroporation. (Since electroporation can be a fairly harsh procedure compared to direct viral infection the cells subject to electroporation are examine for viability. Viability of the IPSC culture cells is determined by their ability to exclude vital dye and analysis of injury-associated cell products such as glycosaminoglycans and hydroperoxides.)
30 Secondary cultures of the transfected cells are established. Re-cultured cells are selected for resistance to phleomycin or neomycin, respectively.

5 b) Identification of Pro-viral DNA in Transformed Cells:

Neomycin or phleomycin resistant cultured cells are tested for the presence of the appropriate transfecting viral DNA. Cells are removed from the culture flasks using C-PEG buffer and digested in lysis buffer containing proteinase K. DNA is phenol/chloroform extracted, then precipitated in ethanol/sodium acetate. Proviral DNA is identified using nested PCR. For the first reaction, PCR primers are used which amplify the entire open reading frame of the appropriate resistance gene. For the second PCR reaction, the PCR product is used as template. Selected internal 5' and 3' primers are used which amplify an internal sequence of known base pair size. The final PCR product is detected by ethidium bromide staining of agarose gels following electrophoresis and/or probing of Southern blots.

10 c) Stability of Transformation: The long-term stability of the transformations is determined by maintaining long-term growing cultures of the transfected cells and periodically testing them for the presence of pro-viral DNA, as described above.

20 These studies provide information on the efficacy and reproducibility of transfection procedures using iPSCs as target cells. Furthermore, they establish a second foundation for use of transformed iPSCs in treating IDD patients.

25 Example 12 - Encapsulation of *In Vitro* Generated Islets and Implantation Into a Mammal

30 Methods for encapsulation of cells are well known in the art (see, for example, Altman *et al.*, 1984, Trans. Am. Soc. Art. Organs, 30:382-386, herein incorporated by reference, in which human insulinomas were enclosed in selectively permeable macrocapsules). Accordingly, isolated *in vitro* generated islets, optionally genetically modified according to Example 11, or pancreas-like structures produced according to examples 3 and 4, are

5 encapsulated in an insulin, glucagon and somatostatin permeable
encapsulant. Preferably such encapsulant is hypoallergenic, is easily and
stably situated in a target tissue, and provides added protection to the
implanted structure such that differentiation into a functional entity is
assured without destruction of the differentiated cells.

10 It should be understood that the examples and embodiments
described herein are for illustrative purposes only and that various
modifications or changes in light thereof will be suggested to persons
skilled in the art and are to be included within the spirit and purview of this
application and the scope of the appended claims.

15

5

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5

Claims

1. A method for the *in vitro* growth of islet producing stem cells, IPSCs, to produce islet cells or an islet-like structure which comprises culturing pancreatic cells from a mammalian species in a basal nutrient medium supplemented with normal serum at below about 0.5% and glucose at below about 1 mM, allowing said IPSCs to grow for at least about 3 weeks, and initiating cellular differentiation into mature islet cells by re-feeding the IPSCs in culture with a nutrient medium supplemented with normal serum at about 0.5-10% and glucose at about 2.5 mM-10 mM.

15

2. The method, according to claim 1, wherein the pancreatic cells are human islet cells and the serum is normal human serum.

3. The method, according to claim 1, wherein the pancreatic cells are mouse islet cells and the serum is normal mouse serum.

20

4. A method, according to claim 1, wherein said nutrient medium comprises a high amino acid nutrient medium.

25

5. The method, according to claim 1, wherein the culture medium used to re-feed said cell culture further comprises glucose.

6. The method, according to claim 1, wherein differentiation of cultured stem cells is initiated at about 4 to 5 weeks of culture growth by re-feeding of said pancreatic cell culture with the nutrient medium supplemented with homologous normal serum.

30

5 7. The method, according to claim 1, wherein after cell differentiation is initiated by re-feeding the culture, the culture is re-fed at about one-week intervals.

10 8. The method, according to claim 1, wherein the normal serum is obtained from the same mammalian species from which the islet cells were obtained.

15 9. The method, according to claim 1, wherein an islet-like tissue structure is produced after differentiation of said IPSCs.

10 10. An islet cell produced by the method of claim 1.

15 11. An islet-like tissue structure produced by the method of Claim 9.

20 12. A method for producing an endocrine hormone which comprises culturing pancreatic cells according to claim 1, and recovering said endocrine hormone from said pancreatic cell culture.

25 13. The method, according to claim 12, wherein said hormone is a human hormone.

30 14. The method, according to claim 12, wherein said hormone is a mouse hormone.

 15. The method, according to claim 12, wherein differentiation is initiated at about 4 to 5 weeks of culture growth by re-feeding of said IPSC cell culture with said nutrient medium supplemented with normal serum.

5 16. The method, according to claim 12, wherein said endocrine hormone is selected from the group consisting of insulin, glucagon and somatostatin.

10 17. A method for producing a pancreas-like organ in a mammal which comprises implanting an islet cell or an islet-like structure produced by the method of claim 1 into the tissue of the mammal.

15 18. A method for treating pancreatic disease in a mammal which comprises producing a pancreatic-like organ in the mammal *in vivo* according to the method of claim 17.

20 19. A pancreas-like organ produced by implanting an islet-like structure into a mammal wherein said islet-like structure is produced by growing islet producing stem cells, IPSCs, present in pancreatic cells from a mammalian species in a basal nutrient medium supplemented with normal serum at below about 0.5% and glucose at below about 1 mM, allowing said IPSCs to grow for at least about 3 weeks, and initiating cellular differentiation into mature islet cells by re-feeding the IPSCs in culture with a nutrient medium supplemented with normal serum at about 0.5-10% and
25 glucose at about 2.5 mM-10 mM.

30 20. The method, according to claim 1, wherein the islet-like structure comprises cells selected from the group consisting of α cells, β cells and δ cells.

21. The method, according to claim 17, wherein said islet-like structure or islet cell implanted into the mammal is autologous to the mammal receiving the implant.

5 22. The method, according to claim 17, wherein the mammal is a human.

 23. The pancreas-like organ, according to claim 19, wherein said organ is produced in a human.

10

 24. A mammal, other than a human, having a pancreas-like organ produced according to the method of claim 17.

15

 25. A mammal, according to claim 24, wherein said mammal is a mouse.

 26. A method for producing a pancreas-like organ in a mammal which comprises implanting at least one pancreatic stem cell into the tissue of said mammal.

20

 27. A pancreas-like organ in a mammal produced according to the method of claim 26.

25

 28. The method of claim 26 in which said at least one pancreatic stem cell has been modified such that it does not express insulin dependent diabetes associated autoantigens, other than insulin, or which has been modified so that it does not express human leukocyte antigens, as said stem cell differentiates into said pancreas-like organ.

30

 29. The method of claim 26 in which said at least one pancreatic stem cell is encapsulated in an insulin, glucagon, somatostatin and other pancreas produced factor permeable capsule.

5 30. A method for analyzing the differentiation of pancreatic stem cells which comprises culturing at least one pancreatic stem cell *in vitro*.

 31. The method of claim 30 which further comprises inducing
10 said at least one stem cell to begin differentiation into a pancreas-like structure.

 32. The method of claim 31 which further comprises identifying
15 mRNA or protein markers specific to a plurality of different stages in the differentiation process.

 33. The method of claim 32 wherein said protein markers are
 expressed on the cell-surface, are secreted, or are intracellular.

 34. A method of making a ligand-binding molecule which
20 selectively binds to pancreatic stem cells or to more differentiated pancreatic cells which comprises contacting a naive B-lymphocyte or T-lymphocyte with a protein marker identified according to the method of claim 33, and culturing and expanding said B-lymphocyte or T-lymphocyte to obtain a population of cells which produce said ligand-binding molecule.

25 35. A ligand-binding molecule prepared according to the method of claim 34.

 36. The ligand-binding molecule of claim 35 which is an
30 antibody, a monoclonal antibody, or a T-cell receptor.

 37. A method of isolating a pancreatic stem cell or a partially differentiated pancreatic cell at any stage between that of a pancreatic stem

5 cell and a fully differentiated pancreatic cell, which comprises selecting said cell from a population of cells comprising said cell, with the ligand-binding molecule of claim 35 which binds to a cell-surface protein marker expressed by said cell at said stage of differentiation, or selecting and removing other
10 molecule of claim 35 which binds to a cell-surface protein marker absent on the surface of said cell.

38. An isolated cell, isolated according to the method of claim 37.

15 39. An isolated pancreatic stem cell or a population of isolated pancreatic stem cells.

40. A method for treating a mammal suffering from, or at risk of
IDD, which comprises:

- 20 a. removing pancreatic tissue from said mammal;
b. culturing pluripotent pancreatic cells present in said pancreatic tissue, *in vitro*, to generate islet-like structures; and
c. implanting said islet-like structures into said mammal.

25 41. An IPSC modified so as not to express insulin dependent diabetes autoantigens in either the undifferentiated or in the differentiated state of the IPSC.

30 42. The modified IPSC of claim 41 wherein said autoantigen which is not expressed as a result of the modification is selected from GAD, 64 kD islet cell surface antigen, and HLA markers.

5 43. The process of claim 5 wherein differentiation is enhanced by inclusion of about 10-25 mM glucose, hepatocyte growth/scatter factor, keratinocyte growth factor, fibroblast growth factor, epidermal growth factor, insulin-like growth factor, nicotinamide, or autocrine growth factors produced by IPSCs.

10 44. The method of claim 43 in which the glucose concentration in the re-fed medium is about 16.7 mM.

15 45. A method for *in vitro* neogenesis of islets from pluripotent stem or progenitor cells which comprises:

- 20 a. establishing a stromal, or "nurse", cell monolayer of ductal pancreatic epithelial cells which permits the generation of IPSCs;
- b. inducing stem/progenitor cell proliferation with culture conditions which promote cyclical regeneration of IPSCs, and also prevent premature differentiation of the IPSCs; and
- c. expanding and differentiating the IPSCs to produce an islet-like structure comprising α , β , and δ cells.

25 46. A method for long-term propagation of IPSCs which comprises serially transferring epithelial cells plus early-stage, proliferating islet-like structures in culture *in vitro*.

30 47. A culture-generated islet-like structure characterized by large, differentiated cells which stain with insulin-specific stain in the center of the islet-like structure; small differentiated cells which stain with glucagon-specific stain at the periphery; and proliferating and undifferentiated cells which do not stain with any of the endocrine hormone-specific stains in the inner cortex; said structure being further characterized in that, upon

5 breaking said structure into single cell suspensions by mechanical means in the presence of a proteolytic enzyme and subsequent staining of individual cells, individual cell populations which stain either with glucagon-specific stain (α cells), insulin-specific stain (β cells) or somatostatin-specific stain (δ cells) are observed.

10

48. The method of *in vitro* neogenesis of islets according to claim 45 which comprises:

a. dispersing and leaving undisturbed pancreatic cells in a minimal culture medium comprising little or no glucose, serum at a concentration below about 0.5%, essential amino acids for the cells of the species from which the pancreatic cells were obtained, and a rudimentary lipid source, until about 99% of the cells in said culture have died (Phase I);

b. re-feeding the culture of step (a) with said minimal medium supplemented with about 1-10 mM glucose and about 0.5-10% serum (but less than a toxic amount of) and re-feeding about once a week until rapid proliferation occurs;

c. re-feeding the culture of step (b) with said minimal medium supplemented with said 0.5-10% serum and about 10-25 mM glucose and, optimally, added growth or cellular factors (Phase III);

d. allowing islet-like structures to bud into the medium;

e. recovering said islet-like structure.

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Figure 1A



Figure 1B

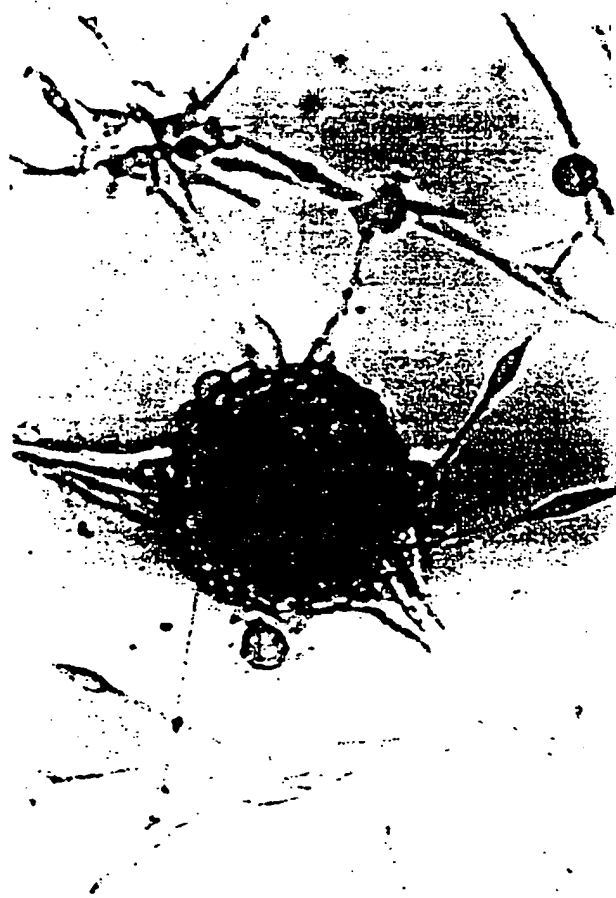


Figure 1C

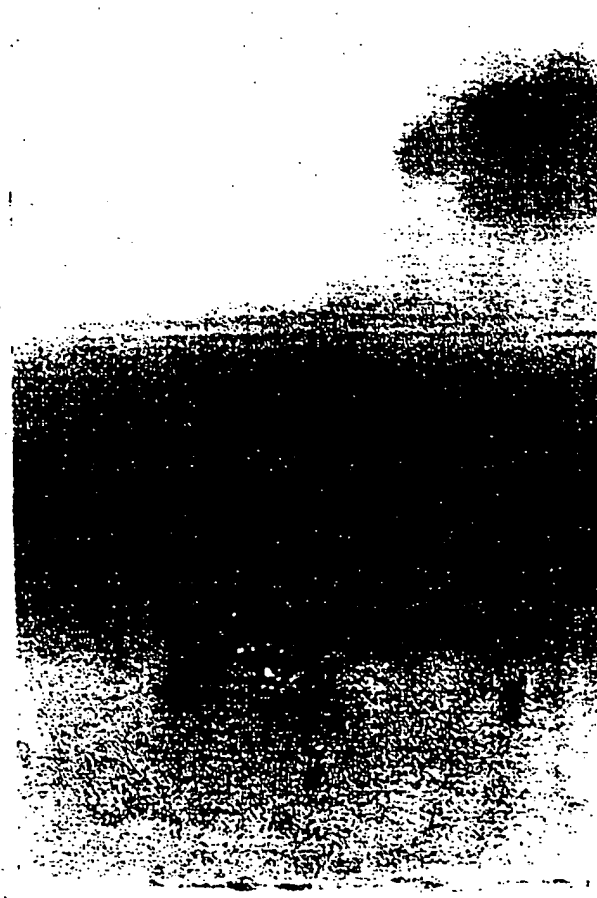


Figure 2

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Figure 1D



FIG. 3B



FIG. 3A



FIG. 3D

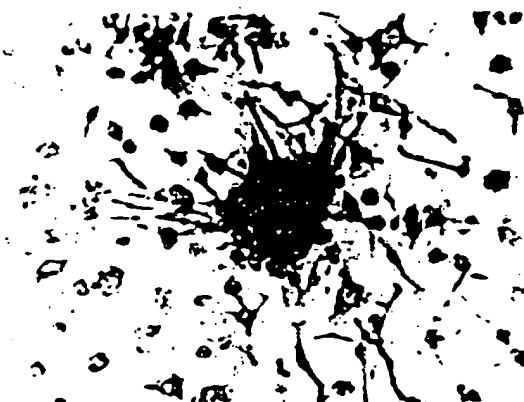


FIG. 3C

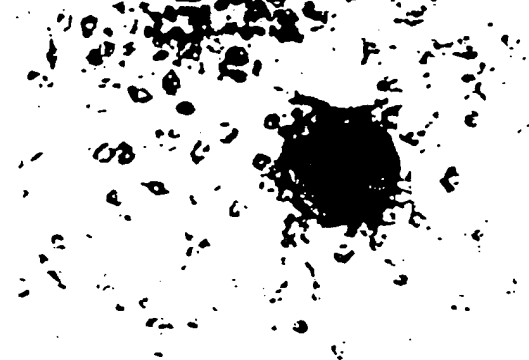


FIG. 3F



FIG. 3E



FIG. 3H

FIG. 3G

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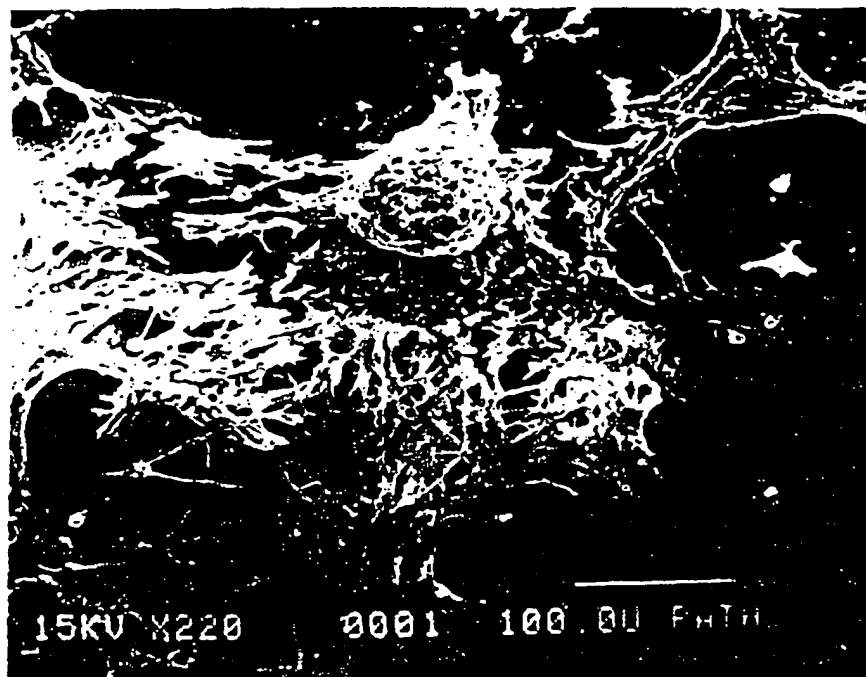


FIG. 4A

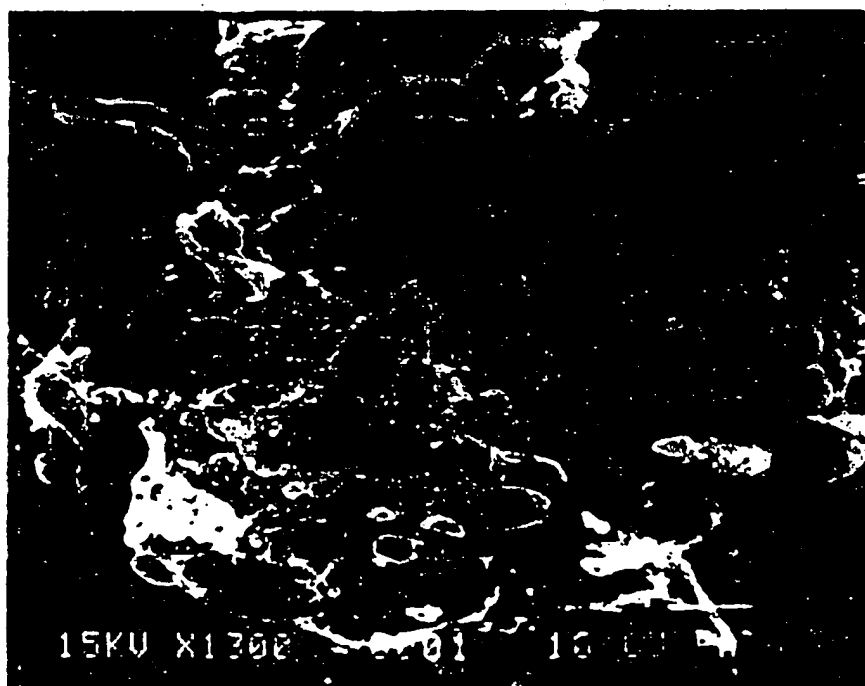


FIG. 4B

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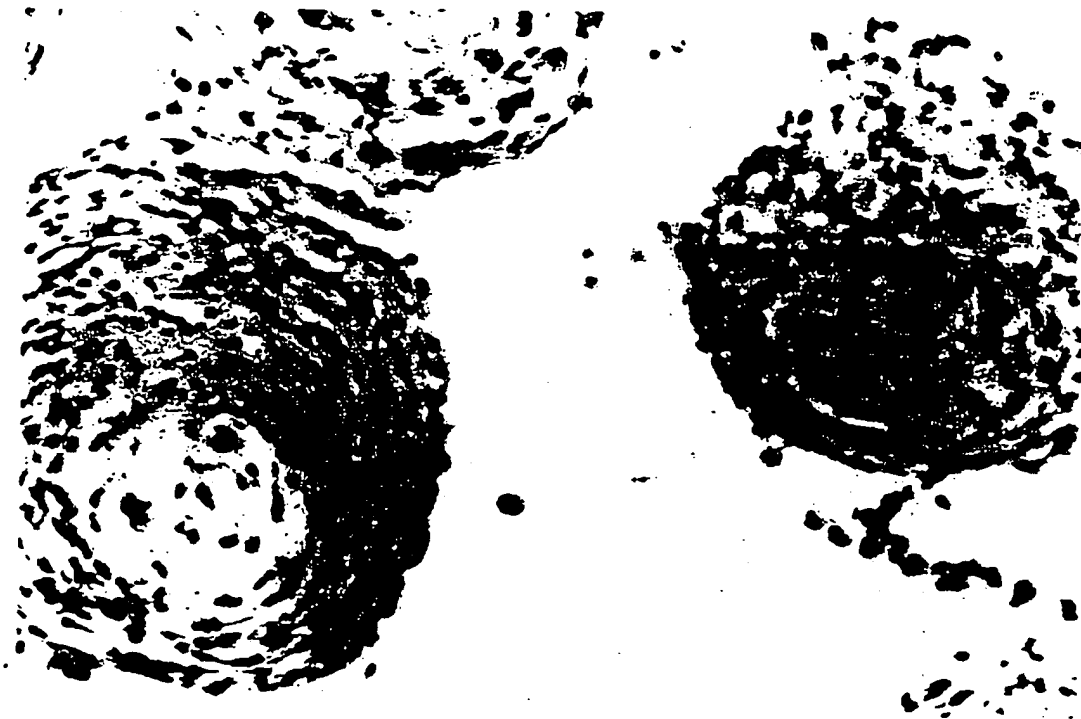


FIG. 5

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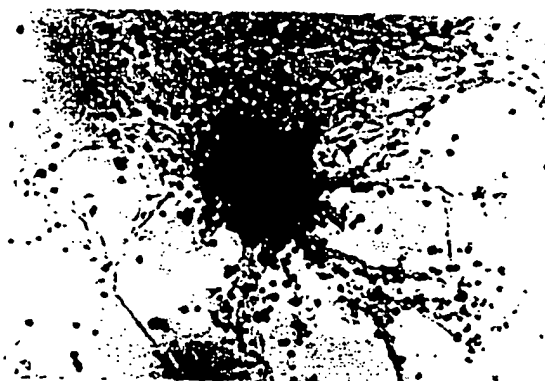


FIG. 6A



FIG. 6B

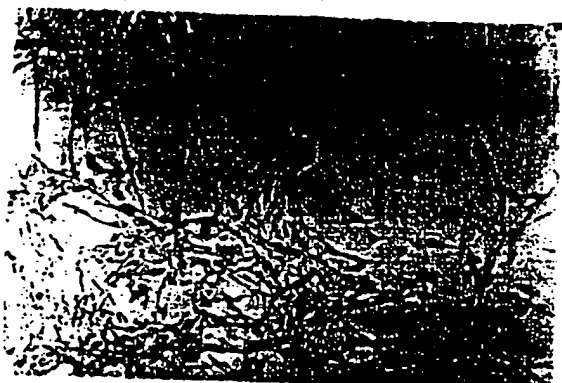


FIG. 6C



FIG. 6D

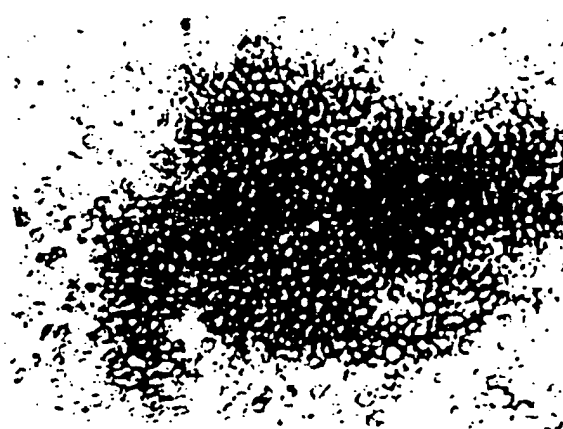


FIG. 6E

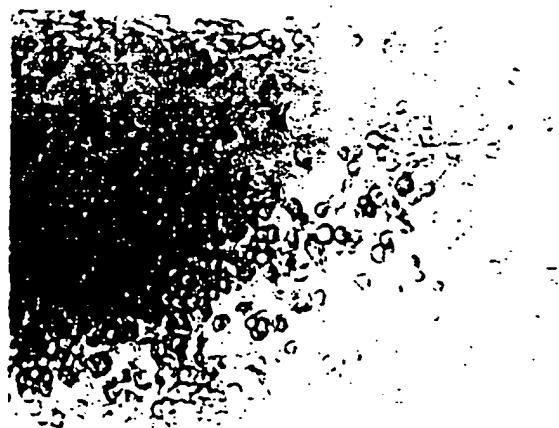


FIG. 6F

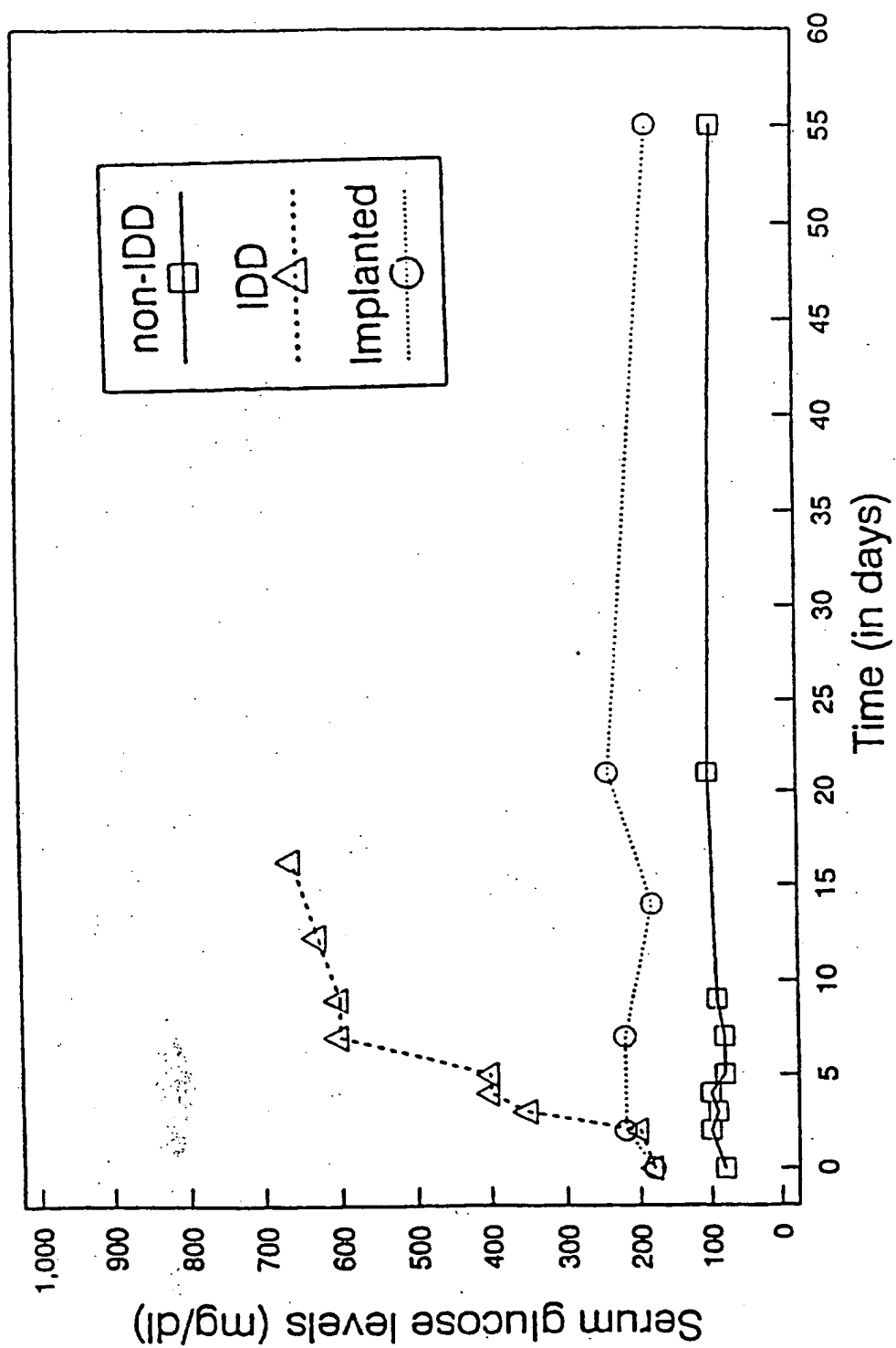


Figure 7

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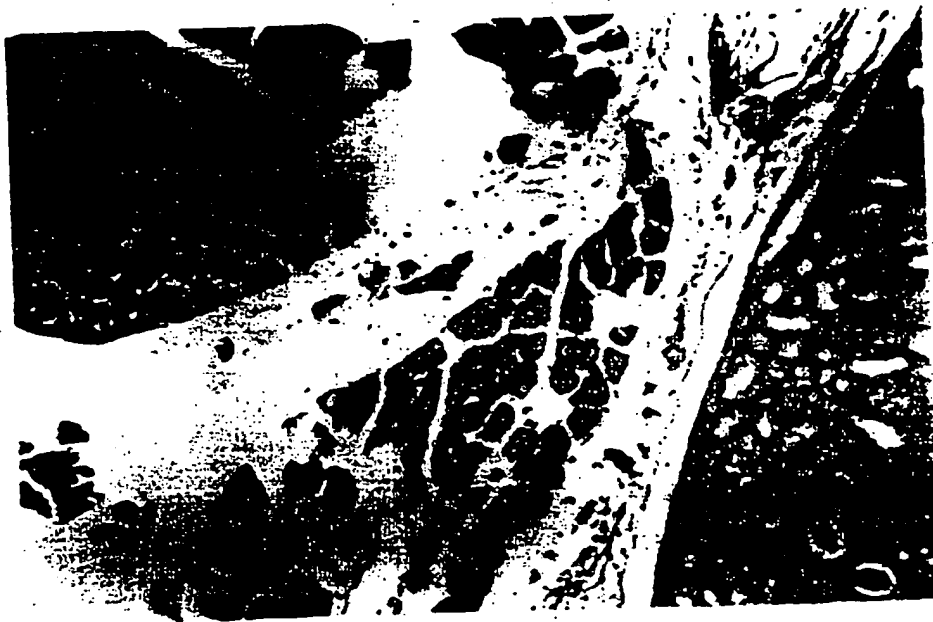
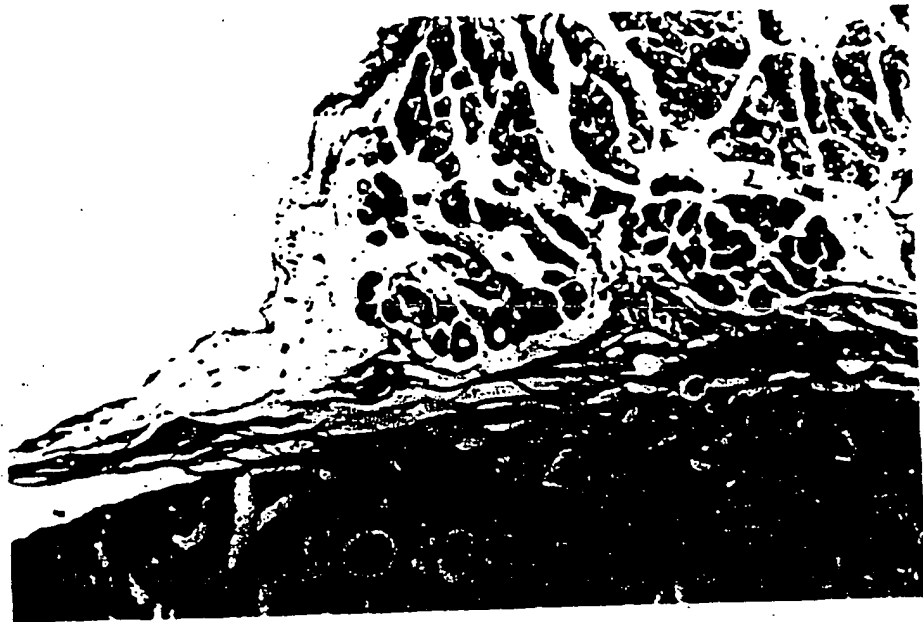


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/16952

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 424/9.1, 93.1, 93.2, 93.21, 130.1; 435/ 4, 69.1, 240.2, 240.21; 514/2, 44; 530/350, 387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1, 93.1, 93.2, 93.21, 130.1; 435/ 4, 69.1, 240.2, 240.21; 514/2, 44; 530/350, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: APS, Medline, CA, Biosis

Search Terms: Peck7/au; Cornelius7/au; pancre7; islet7; cultur7; stem; diabet7; insul7; organ; develop; vitro; vivo;

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-------------|--|--|
| X — Y | WO,A, 93/00441 (PACIFIC BIOMEDICAL RESEARCH INC.) 07 January 1993, see entire document. | 10, 12-16, 20, 38, 43, and 44 1-9, 26, 28-34, 36, 37, 40-42, and 45-48 |
| X — Y | WO,A, 94/23572 (HUMAN CELL CULTURES, INC.) 27 October 1994, see entire document. | 11, 17-19, 21- 25, and 27 1-9, 26, 28-34, 36, 37, 40-42, and 45-48 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | |
|---|----|--|
| * Special categories of cited documents: | T | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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| *E* earlier document published on or after the international filing date | Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | &* | document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | | |
| *P* document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

15 JANUARY 1997

Date of mailing of the international search report

11 FEB 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16952

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|--------------|--|---|
| X <hr/> Y | KANAKA-GANTENBEIN et al., "Presence of nerve growth factor and its receptors in an in vitro model of islet cell development: implication in normal islet morphogenesis", Endocrinology. 1995, Vol. 136, No. 7, pages 3154-3162, see entire document. | 35 and 39 <hr/> 1-9, 26, 28-34, 36, 37, 40-42, and 45-48 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/16952

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 31/70, 38/00, 49/00; A01N 43/04, 63/00, 65/00; C12N 5/00, 5/06, 5/10, 5/16, 5/18, 5/22, 15/06, 15/07; C12Q 1/02